



Role of Toll-like receptors in immune activation and tolerance in the liver

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Liver has a unique vascular system receiving the majority of the blood supply from the gastrointestinal tract through the portal vein and faces continuous exposure to foreign pathogens and commensal bacterial products. These gut-derived antigens stimulate liver cells and result in a distinctive immune response via a family of pattern recognition receptors, the Toll-like receptors (TLRs). TLRs are expressed on Kupffer cells, dendritic cells, hepatic stellate cells, endothelial cells, and hepatocytes in the liver. The crosstalk between gut-derived antigens and TLRs on immune cells trigger a distinctive set of mechanisms to induce immunity, contributing to various acute and chronic liver diseases including liver cirrhosis and hepatocellular carcinoma. Accumulating evidence has shown that TLRs stimulation by foreign antigens induces the production of immunoactivating and immunoregulatory cytokines. Furthermore, the immunoregulatory arm of TLR stimulation can also control excessive tissue damage. With this knowledge at hand, it is important to clarify the dual role of disease-specific TLRs as activators and regulators, especially in the liver. We will review the current understanding of TLR signaling and subsequent immune activation and tolerance by the innate immune system in the liver.

Keywords: Toll-like receptor, Kupffer cell, dendritic cell, liver tolerance, microbiota

INTRODUCTION

The liver faces continuous exposure to many pathogens and commensal bacterial products, and the innate and adaptive immune responses of the liver favor the induction of immunological activation and tolerance as appropriate (1–5). Although various immune compartments, such as T cells including CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), natural killer (NK) cells, natural killer T (NKT) cells, macrophages [Kupffer cells (KC)], conventional or classical dendritic cells (cDCs), and plasmacytoid DCs (pDCs), reside in the normal liver (1, 2), it is unknown which types of cells induce inflammation and tolerance and how these cells work together to maintain immunological balance. The innate immune system is thought to play a major role in maintaining homeostasis in the liver. Gut-derived bacterial products enter the liver through the portal vein. However, liver inflammation usually does not occur because the intact mucosal barrier system of the healthy intestine prevents translocation of microbial products. When this barrier is disrupted, bacteria translocate to the liver in large quantities, resulting in the activation of the hepatic innate immune system. Cells within the hepatic sinusoids express a receptor that recognizes lipopolysaccharide endotoxin (LPS), expressed in the outer membrane of Gram-negative bacteria, and effectively remove this molecule. The Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) as part of innate immune defenses against foreign pathogens, including bacteria, DNA and RNA viruses, fungi, and protozoa (6, 7). Thirteen mammalian TLRs have been identified, and TLR1–10 are expressed in humans. TLRs, their ligands, and downstream signaling pathways are shown in **Figure 1**. In general, the healthy liver contains low mRNA levels of TLRs and their

downstream signaling molecules, such as myeloid differentiation primary response gene-88 (MyD88), compared with other organs (8, 9). The continuous antigen exposure and recognition via TLRs in the liver may trigger a distinctive set of mechanisms to maintain self-tolerance and induce immunity against infection depending on the particular situation. Here, we will review the dual role of TLRs as activators and regulators of immune responses in the liver.

TLR SIGNALING IN THE LIVER

In the liver, hepatocytes account for 60–80% of the total cell population (10). Non-parenchymal cells consist of KCs, DCs, lymphocytes, hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSECs), and biliary cells. Each cell population exhibits a different TLR expression.

KUPFFER CELLS

Kupffer cells are hepatic-resident macrophages and account for about 20% of the non-parenchymal cells in the liver. KCs engage in phagocytosis and antigen presentation, and they are the primary cells that encounter gut-derived toxins such as LPS and orchestrate immune responses within the liver (11). Accordingly, KCs express TLR4 and are responsive to LPS (12). KCs also express TLR2, TLR3, and TLR9 and respond to their ligands (13–15). Following LPS stimulation, KCs produce tumor necrosis factor α (TNF α), interleukin (IL)-1 β , IL-6, IL-12, and IL-18 (16). However, KCs also release anti-inflammatory cytokines such as IL-10 in response to continuous stimulation with low levels of LPS, a phenomenon known as LPS tolerance (17). Similarly, several murine experiments demonstrated a role for macrophages in restricting inflammatory responses during the recovery phase of liver injury

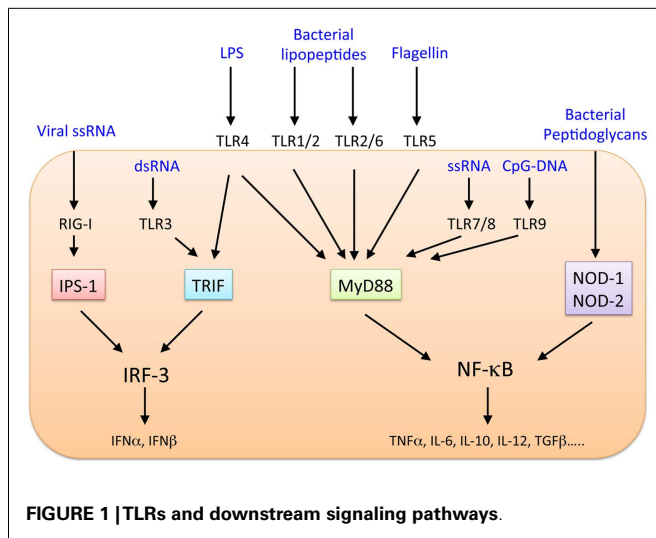


FIGURE 1 | TLRs and downstream signaling pathways.

(18). These results indicate that KCs act as both immune activating and immune regulatory cells depending on the specific situation.

DENDRITIC CELLS

Hepatic DCs account for a very small proportion (<1%) of non-parenchymal cells in the liver. DCs in lymphoid and non-lymphoid tissues are classified into two major subsets: pDCs and cDCs. Murine lymphoid-resident cDC subsets can be further divided into CD8 α ⁺ DCs and CD8 α ⁻ DCs (19, 20). Likewise, two distinct migratory cDC subsets are subcategorized based on CD103 and CD11b expressions in non-lymphoid tissues: CD103⁺CD11b⁻ cDCs and CD103⁻CD11b⁺ cDCs (21, 22). In humans, pDCs express TLR1, TLR7, and TLR9, while other DC subsets express all other TLRs except for TLR9 (23). In mice, both pDCs and cDCs express TLR2, TLR4, TLR7, and TLR9. In response to signaling through TLR2, TLR3, and TLR4, hepatic cDCs produce TNF α and IL-6 (24). However, recent reports showed that murine cDCs can produce an anti-inflammatory cytokine, IL-10, through TLR9 following ischemia/reperfusion injury (25). Hepatic pDCs produce inflammatory cytokines in response to TLR7 and TLR9 (24, 26, 27). Of note, a new subset of CCR9⁺ pDCs was identified as tolerogenic pDCs in an acute graft-versus-host disease model (28). Our group demonstrated CCR9⁺ pDCs exist abundantly within the murine liver, produce IL-10, and transforming growth factor β (TGF β) and differentiate naive T cells to a regulatory phenotype through TLR7 and TLR9 signaling (29).

LYMPHOCYTES

Intrahepatic lymphocytes account for about 25% of the non-parenchymal cells in the liver. They consist of NK, NKT, $\gamma\delta$ T, $\alpha\beta$ T, and B cells. Hepatic NK cells express TLRs 1, 2, 3, 4, 6, 7, 8, and 9 and respond to the corresponding TLR ligands (30). TLR3 ligands negatively regulate liver regeneration via activation of NK cells (31). In general, T cells are indirectly activated by TLR signaling, but direct activation of T cells by TLR signaling through TLR2, 3, and 9 has been reported (32, 33).

HEPATIC STELLATE CELLS

Hepatic stellate cells account for a very small proportion (<1%) of non-parenchymal cells in the liver. Following liver injury, activated HSCs produce extracellular matrix components in the liver, such as collagen types 1, 3, and 4, leading to liver fibrosis (34). Activated human HSCs express TLR4 and CD14, and respond to LPS with the secretion of proinflammatory cytokines (35). Activated mouse HSCs express TLR2, TLR4, and TLR9, and respond to the corresponding ligands with the secretion of IL-6, vascular cell adhesion molecule 1 (VCAM-1), TGF β 1, and monocyte chemoattractant protein-1 (MCP-1) (36–38).

LIVER SINUSOIDAL ENDOTHELIAL CELLS

Liver sinusoidal endothelial cells account for about 50% of non-parenchymal cells in the liver. LSECs express mRNAs for TLR1–9 and respond to the corresponding ligands except for that of TLR5. LSECs respond to TLR1, 2, 4, 6, 9 ligands by producing TNF α , and respond to TLR3 ligands by producing TNF α , IL-6, and interferon (IFN) (27). After repetitive LPS challenge, sinusoidal endothelial cells reduce NF- κ B activation and mediate liver tolerance to maintain hepatic homeostasis (39). In the same way, LSECs play a role in maintaining the homeostasis of the liver through induction of antigen-specific T cell tolerance (40). A recent report demonstrated that LSECs mediate angiogenesis and subsequent liver fibrosis via TLR4 signaling (41).

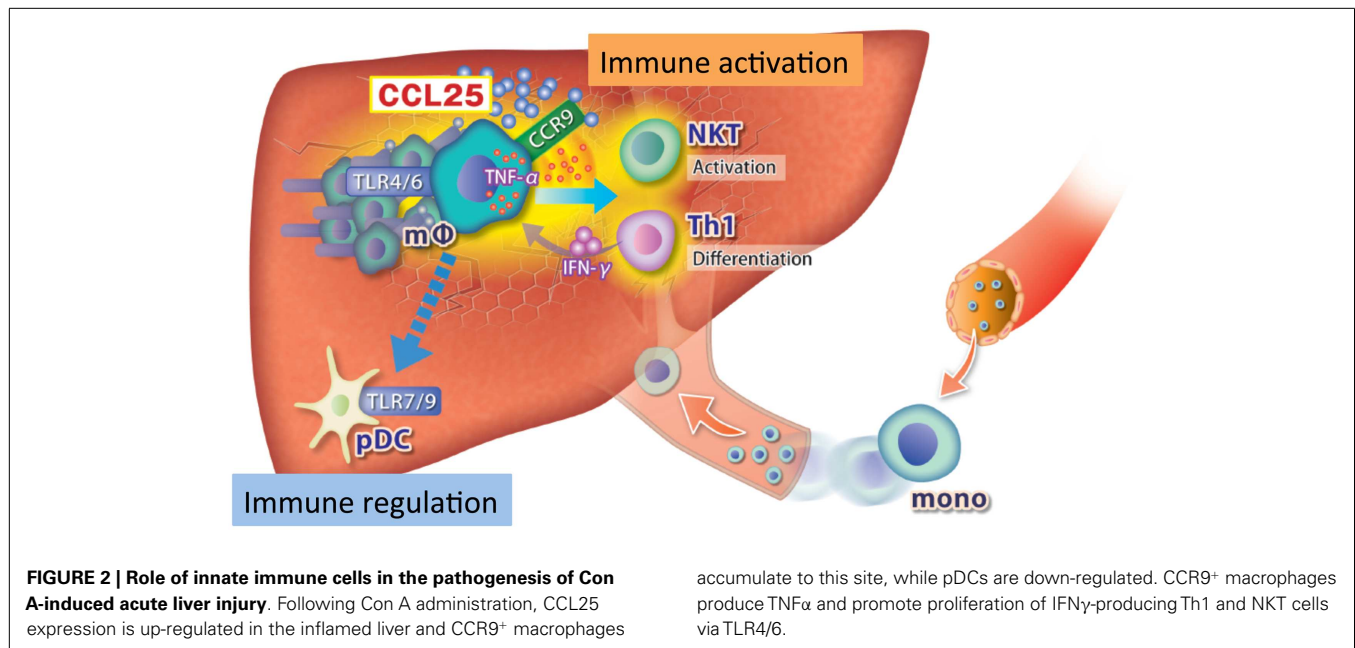
HEPATOCYTES

Primary cultured hepatocytes express TLR1–9, but only respond to TLR2 and TLR4 ligands (42). In the steady state, the responses to TLR2 and TLR4 are weak, while the expression of TLR2 and responsiveness to ligands is enhanced under inflammatory conditions (43, 44). Of note, hepatocytes, in concert with TLR4, CD14, and MD-2 play a role in the uptake and removal of LPS from the systemic circulation (45–47).

ROLE OF TLRs IN MURINE AND HUMAN LIVER INJURY

EXPERIMENTAL ACUTE LIVER INJURY (CONCANAVALIN A)

A single intravenous injection of Con A triggers acute liver injury in mice. It is accepted that Con A-induced acute liver injury is mediated mainly by CD3⁺CD4⁺NK1.1⁺NKT cells and CD3⁺CD4⁺NK1.1⁻ T cells (48–50). However, liver antigen-presenting cells (APCs) including KCs and DCs might be critically involved in the pathogenesis of Con A-induced liver injury, since it is significantly suppressed in KC-depleted mice (51–53). Signaling through TLR2, TLR3, TLR4, and TLR9 has been reported to contribute to liver injury in this model, especially in the early phase (54–56). We recently reported that TNF α -producing CCR9⁺CD11b⁺CD11c⁻ macrophages expressed TLR2, TLR4, and TLR6 mRNAs and had a major role in the pathogenesis of acute liver injury in this model by activating Th1 and NKT cells (25). Of note, in the inflamed liver the number of tolerogenic CCR9⁺CD11b⁻CD11c⁺ pDCs that express TLR7 and TLR9 mRNAs decreases following Con A injection, suggesting the balance between inflammation and tolerance might be regulated by distinct immune cell subsets and TLRs in this model (Figure 2). Following Con A injection, up-regulation of TLR3 expression is observed in liver mononuclear cells and LSECs. The pathological role of TLR3 in this model was confirmed as TLR3^{-/-} mice



were protected from Con A-induced hepatitis (57). In contrast, it was reported that Poly-I:C pretreatment activated NK cells and subsequently protected against Con A-mediated liver injury via down-regulation of T/NKT cells (58). Importantly, the protective effect of TLR3 was also reported in an LPS/D-GalN-induced acute liver injury model (14). These results collectively indicate that TLR3 signaling has pleiotropic functions and is involved in inflammation, regeneration, and tolerance during the course of acute liver injury. The contribution of TLR9 in this model is controversial. TLR9 activation by CpG oligodeoxynucleotides (CpG-ODN) can exacerbate Con A-induced liver injury by promoting the activation of hepatic CD4⁺ NKT cells. The effect of TLR9 signaling on hepatic NKT cells was dependent on KCs and IL-12 (59). However, another report showed that pretreatment with CpG-ODN protected mice from Con A-induced hepatic injury by attenuating the activation of inflammatory cells (60). These contradictory findings could have resulted from differences in the DNA sequences used, because a different DNA sequences might trigger TLR9 signaling with different consequences, such as the release of potentially harmful (TNF α) or beneficial (IL-12) cytokines (61). Immunological tolerance to Con A was demonstrated as repeated Con A injection within 8 days after an initial Con A injection significantly reduced hepatic injury (62). The authors of that study concluded that CD4⁺CD25⁺ Tregs, KCs, and IL-10 were required for Con A tolerance. Further studies are required to clarify the contribution of specific TLRs and their downstream signaling to Con A tolerance.

ISCHEMIA–REPERFUSION AND LIVER TRANSPLANTATION

Ischemia–reperfusion (I/R) injury is most commonly seen in the early period after liver transplantation. Recipients transplanted with livers from TLR4-deficient mice exhibited less I/R injury than those transplanted with wild-type livers (63), suggesting the inflammatory response seen in I/R injury is mainly mediated by

TLR4. An alternative explanation is that TLR4 plays an indirect role by exacerbating I/R, as opposed to initiating the pathology. TLR4 expression on non-parenchymal cells is up-regulated by damage-associated molecular pattern molecules (DAMPs), such as high-mobility group box 1 protein (HMGB1) released from damaged hepatocytes during I/R (64, 65). Regarding allograft rejection and tolerance in liver transplantation, hepatic TLR4 expression has a distinctive role in CD8 T cell apoptosis and memory T cell generation (66). Increased TLR4-mediated expression of adhesion molecules in LSECs and KCs following continuous LPS exposure promoted trapping of T cells within the liver, resulting in lower numbers of circulating primed CD8 T cells and weak immune responses (39). The balance between alloimmune responsiveness and tolerance might be mediated by the level of TLR ligands that act as PAMPs or DAMPs, in association with clinical events such as I/R injury and infection after transplantation (67). It was recently reported that IL-10-producing cDCs reduced liver I/R injury in mice via TLR9 (25). Although the liver can mount an appropriate and sometimes excessive immune response to eliminate invading organisms, the overall balance appears to favor a state of immune permissiveness. As critical regulators of both innate and adaptive immunity, hepatic cDCs might play a role in orchestrating immune responses to limit undesirable inflammation and promote tolerance via TLR9. It is still unclear how the immune system can distinguish between threats from pathogens and endogenous danger signals, and contribute to both immune activation and tolerance through TLR9 signaling.

HEPATITIS B AND HEPATITIS C VIRAL INFECTION

The gene expression of TLR1, TLR2, TLR4, TLR6, TLR7, and TLR9 was decreased in peripheral blood mononuclear cells (PBMCs) from chronic Hepatitis B virus (HBV) infected patients, compared with healthy controls (68, 69). Impaired cytokine production with TLR2 and TLR4 ligands was also observed in PBMCs from chronic

Hepatitis B (CHB) patients (68). Several TLR signaling pathways induce antiviral effects by up-regulating IFNs. Activation of TLR3, TLR4, TLR5, TLR7, and TLR9 by ligands mediates the inhibition of viral replication in HBV transgenic mice (70, 71). Importantly, HBV infection also induces immunosuppressive effects through TLR signaling. Overexpression of TLR2 and TLR4 on monocytes is reported to account for persistent HBV infection by modulating Treg functions (72). In PBMCs from chronic Hepatitis C virus (HCV) infected patients, the gene expression of TLR2, TLR3, TLR4, TLR6, TLR7 was increased (73, 74). HCV activates innate immune receptors including TLRs and retinoic acid-inducible gene 1 (RIG-I) to induce a chronic inflammatory state. Concurrently, HCV suppresses specific intracellular signaling to evade the host immune control (75). HCV core and NS3 proteins trigger TLR1, TLR2, and TLR6 on monocytes to enhance the production of inflammatory cytokines (76, 77). However, NS3/4a proteins degrade TIR-domain-containing adapter-inducing IFN- β (TRIF) and inhibit TLR3-mediated TRIF-dependent IFN- β production (78, 79). Furthermore, NS5 inhibits the recruitment of IL-1 receptor-associated kinase 1 (IRAK1), resulting in a decrease in TLRs-Myd88-dependent signals (80). An appropriate T cell response is required to eradicate HBV and HCV, while exhausted HCV-specific T cells with inhibitory immune receptors, such as PD-1 and CTLA-4, account for persistent viral infection within the liver (3, 4, 81, 82). LSECs with up-regulated PD-L1 expression were reported to induce antigen-specific T cell tolerance (40), and recent reports indicated that stimulation of LSECs with TLR1/2 ligands, but not TLR3 or TLR4 ligands could overcome liver-specific tolerance (83). Further study is required to clarify the effect of TLR1/2 ligands on the function of tolerant HBV- and HCV-specific T cells.

ALCOHOL-INDUCED LIVER DISEASE

Excessive alcohol intake induces elevated levels of LPS in the liver through the portal circulation (84). The mechanism involved in the elevation of LPS is thought to be as follows. First, ingested alcohol disrupts the intestinal mucosal barrier and causes enhanced permeability (85, 86). Second, alcohol consumption leads to changes in the intestinal flora (87), and they migrate to liver sinusoids through the portal vein. KCs are a major target of LPS in various liver injuries including alcohol-induced liver injury (35, 88), as demonstrated by reduced liver inflammation following KC depletion (89). Recent reports indicated that TLR4 signaling in alcoholic liver injury was mediated through a MyD88-independent, but TRIF-dependent pathway (90, 91).

NON-ALCOHOLIC STEATOHEPATITIS

Accumulating evidence indicates that LPS/TLR4 is also involved in the development of non-alcoholic steatohepatitis (NASH). A role for LPS in NASH was demonstrated by the finding that genetically obese *ob/ob* mice were sensitive to low-dose LPS (92). Furthermore, when fed a methionine/choline-deficient (MCD) diet, the most widely accepted experiment model of NASH, TLR4-deficient mice exhibited less severe hepatic injury and less accumulation of intrahepatic lipids compared with wild-type mice (93). These findings indicated activated TLR4 signaling pathways were critically involved in the pathogenesis of NASH. Recently, up-regulation of CD14 in KCs and hypersensitivity against low-dose LPS were

observed in mice with high-fat diet (HFD)-induced steatosis (94). Hypersensitivity against low-dose LPS leads to accelerated NASH progression, including liver inflammation and fibrosis. In contrast, TLR2-deficient mice were not protected from steatohepatitis induced by MCD diet, affirming the TLR4 dependence of disease progression in this model (95). Notably, probiotics relieve the severity of NASH in leptin-deficient *ob/ob* mice, suggesting alterations of the intestinal flora might affect proinflammatory responses by disease-specific immune components through TLRs (96, 97).

LIVER FIBROSIS

Studies demonstrated elevated plasma LPS levels in experimental liver fibrosis induced by carbon tetrachloride (CCl_4), thioacetamide, and bile duct ligation (BDL). TLR4 is expressed on both parenchymal and non-parenchymal cells in the liver, and several animal studies support the contribution of TLR4 in the development of liver fibrosis (36, 98, 99). Mice deficient for TLR4, CD14, MyD88, or TRIF exhibit reduced liver fibrosis in experimental fibrosis models (36, 98). In a recent study, Seki et al., clearly demonstrated that TLR4 on HSCs, but not on KCs or hepatocytes, was crucial for inducing liver fibrosis (36). Low concentrations of LPS can activate HSCs via TLR4 and downstream signaling to secrete a number of chemokines and adhesion molecules. These chemokines not only induce the migration of macrophages into the liver but also directly activate HSCs, leading to liver fibrosis. The role of chemokine receptors CCR1, CCR2, CCR5, CCR8, and CCR9 in liver fibrosis has been reported (100–104). A human study analyzing a large patient cohort demonstrated that certain single nucleotide polymorphisms (SNPs) in TLR4 were associated with reduced risk of liver cirrhosis in patients with chronic hepatitis C (105). The participation of TLR9 during liver fibrosis has been demonstrated in several mouse models of liver fibrosis, such as CCl_4 and BDL models, in which TLR9-deficient mice exhibited significant reductions in liver fibrosis (106). Endogenous DNA from damaged hepatocytes is reported to enhance HSC activation through TLR9, thereby promoting liver fibrosis (37). TLR3 participates in the early stages of liver fibrosis but not during advanced liver fibrosis. Treatment with the TLR3 ligand Poly-I:C enhanced the activation of NK cells for killing HSCs, leading to attenuation of liver fibrosis (107). Recently, impaired TLR3 and TLR7/8 function was reported to affect rapid fibrosis progression post-liver transplantation with HCV infection (108).

TLRS AND MICROBIOTA

The translocation of intestinal microbiota into the liver and their recognition by TLRs results in both immune activation and tolerance under specific conditions. Importantly, this process is also critically involved in the development of a variety of liver diseases (109–112). Thus, targeting components of innate immune signaling, such as intestinal microbiota and TLRs may be an effective therapeutic approach to chronic liver diseases including viral hepatitis, alcoholic liver disease, NASH, and subsequent liver fibrosis. In particular, the mechanism of how endogenous TLR ligands associated with bacterial translocation contributes to immune activation and regulation, and subsequent chronic liver disease, should be comprehensively studied. Recent advances

in gnotobiotic technology have enabled analysis of the role of specific bacterial strains in immunological responses (113–116). Using these techniques, a recent study reported that a complex mixture of 46 strains of *Clostridium* induced TGF β in intestinal epithelial cells, which promoted the subsequent accumulation of IL-10-producing induced T regulatory cells, which in turn suppressed colitis in a dextran sodium sulfate colitis model (117). Very recently, our group reported that a single strain of *Clostridium butyricum* induced intestinal IL-10-producing macrophages via TLR2 and suppressed a mouse model of acute experimental colitis (118). Furthermore, butyrate-producing probiotics reduced the severity of murine NASH (119). These results clearly indicate that a single strain of microbiota can trigger immune activation and regulation via signaling through distinct TLRs. Further research should address in detail the crosstalk between disease-specific microbiota and the innate and adaptive immune system that occurs via specific TLRs signaling pathways in chronic liver diseases.

CONCLUSION AND PERSPECTIVES

The liver is continuously exposed to food antigens and PAMPs from the gastrointestinal tract via the portal vein. TLR signaling has a critical role in maintaining a balance between immune activation and tolerance. Following exposure to foreign antigens, TLRs are immediately activated and promote the induction of inflammatory cytokines and antimicrobial peptides to remove foreign microorganisms from the host. Concurrently, overactivation of TLRs that causes fetal events such as sepsis and acute liver failure should be controlled, which in turn might result in persistent infections in the liver. As described in this review, the following mechanisms have substantial roles in organ-specific tolerance: (1) hyporesponsiveness of individual TLR signaling due to the continuous exposure to ligands as seen in LPS tolerance (TLR4 on macrophages and LSECs) (17, 39), (2) the induction of other TLR signaling by DAMPs and host DNAs released from injured host cells and subsequent immunosuppressive cytokine production as seen in liver I/R injuries (TLR9 on cDCs) (25), and (3) dysfunctional antigen presentation by PD-L1-expressing APCs and the subsequent antigen-specific T cell exhaustion that can be reversed by TLR1/2 ligand stimulation as seen in chronic viral infections (TLR1/2 on LSECs) (83). Further studies, especially in humans, are required to clarify the interaction of each ligand-TLR signaling pathway on individual immune cell subsets that causes both immune activation and tolerance depending on severity and phase of the injury, and which eventually results in liver diseases such as chronic hepatitis, liver cirrhosis, and liver cancer. Understanding the underlying mechanisms in this area can aid the development of new therapeutic strategies in the future.

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